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August, 1976

Semiannual Progress Report No. 8
January 1, 1976 - June 30, 1976

**RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL
PLANETARY ENVIRONMENTS**

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Abilene, Texas



RESPONSE OF SELECTED MICROORGANISMS TO
EXPERIMENTAL PLANETARY ENVIRONMENTS

Semiannual Progress Report No. 8 of
Planetary Quarantine Activities
January 1, 1976 - June 30, 1976

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FOREWORD

This eighth semiannual progress report summarizes work performed for the National Aeronautics and Space Administration by the Science Research Center at Hardin-Simmons University supported by NASA Grant NGR 44-095-001, and covers the period January 1, 1976 - June 30, 1976.

This report includes results on the anaerobic conversion of phosphite to phosphate and demonstrates that in the presence of both phosphite and hypophosphite, the phosphite is the preferred phosphorous source. An investigation in which ^{32}P labeled hypophosphite added to the basal medium demonstrates that the labeled hypophosphite was incorporated into the metabolic reactions of the cell. Other data show that as cell growth occurs, the phosphite and/or hypophosphite levels decrease. The Bacillus sp. capable of anaerobic utilization of phosphite was isolated from Cape Canaveral soil samples, and it is partially characterized in this report.

Also included in this report are continued investigations of omnitherms. The data presented show that some of these possess a significant resistance to the Viking dry-heat cycle, and that they retain their omnithermic characteristic after recovery from the heat cycle. Other physiological characteristics of these isolates are also presented. A repeat of the original work in which omnitherms were isolated again demonstrate that omnitherms can be isolated from Cape Canaveral soil.

The NASA Technical Officer for this grant is Richard S. Young, NASA Planetary Programs, Code SBL, Washington, D.C.

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ANAEROBIC UTILIZATION OF PHOSPHITE

In our previous progress report (No. 7) the importance of anaerobic utilization of phosphite in potential contamination of Jupiter was discussed. Attempts to isolate organisms capable of this type of phosphorous metabolism were described, and a Bacillus sp. was isolated on different occasions which appeared to possess this characteristic. Initial experiments to verify this demonstrated that the isolate is capable of utilizing phosphite or hypophosphite anaerobically. Additional experiments were performed to better demonstrate this phenomenon. Most experiments employed hypophosphite because it is the more reduced form.

One investigation to verify that the isolate was indeed utilizing hypophosphite employed labeled (^{32}P) hypophosphite (Amersham/Searle, Arlington Heights, Illinois). This was performed by using paper chromatography with known samples of hypophosphite, phosphite, and phosphate. The chromatograms were then used to expose x-ray film (Kodak RP X-Omat RP14) showing separation of compounds containing the ^{32}P labeled phosphorus.

A basal medium was prepared according to the following formulation:

0.5% glucose

0.2% NH_4Cl

0.002% NaSO_4

0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

1.2% Tris Buffer

The glucose was made as a separate solution, sterilized by autoclaving

at 118°C for 15 minutes, then aseptically added to components of basal medium. To this a mineral supplement was added as shown in report No. 7. The solution was then adjusted to a pH of 7.0 and analyzed for phosphorus using the stannous chloride reduction method (Report No. 7).

Sodium hypophosphite was prepared and filter sterilized, then added to the basal medium to a final concentration of 75 ppm phosphorus as $\text{NaH}_2\text{PO}_2 \cdot \text{H}_2\text{O}$ and in addition contained 0.20 MC of ^{32}P per mg of ^{31}P . Into each of four flasks of labeled (^{32}P) hypophosphite medium 0.1 ml of a washed, distilled water suspension of the organism shown to grow in hypophosphite was inoculated and immediately placed into an anaerobe jar. Cultures were then incubated for 48 hours at 32°C. At the end of 48 hours the samples were filtered and washed using a 0.22 μm filter. The cells were then resuspended in distilled water and lysed using a high intensity sonic probe. A one-dimensional descending paper chromatographic system was then run on the lysed cell suspension, uninoculated medium, the filtrate, and known samples of the three phosphorus types. The chromatographic paper was a No. 1 Whatman washed with 2N HCl and rinsed 6 times with distilled water and dried in air before use.

Application of the samples was made onto the chromatographic paper using ten 100 microliter deposits at each of six designated spots near the end of the paper. Two chromatograms were run, each containing six sample sites - one each for the three known samples of phosphite, hypophosphite, and phosphate and the other for the filtrate, medium, and the cell lysate. The spotted chromatographic paper was then allowed to air dry.

The dried paper was placed into a chromatographic chamber where the entire system (including the spotted paper) was allowed to come to equilibrium for approximately 24 hr. The solvent system used was n-butanol-acetic acid-water mixture in the ratio of 50-25-25. This solvent was placed in a large shallow dish at the bottom of the chamber. After allowing for equilibrium of solvent throughout the chamber, solvent was added to trough by funnel from above without opening whole chamber. Development time was determined by solvent front and varied from 12 - 24 hours.

The method of Hans and Isherwood (Nature, page 1107, December, 1949) was used to detect phosphate after all spots had been oxidized. The paper was sprayed with a solution of 5 ml 60% (w/w) perchloric acid, 10 ml 1 N HCl, 25 ml 4% ammonium molybdate, diluted with distilled water to 100 ml. The paper was allowed to dry a few minutes to get rid of excess water, then placed in an oven and heated for 7 minutes at 85°C. It was then hung in a chamber with dilute H₂S to develope the spots. Spots appear blue against a buff background.

Known samples of fructose-1, 6-diphosphate, phosphoenolpyruvate, 2-phosphoglycerate, hypophosphite, phosphite, phosphate were also run on seperate chromatograms as controls. Table 1 shows the results of the control chromatogram. Separation was good with spots easily seen.

Before the chromatograms were oxidized and spots detected, autoradiograms were made. Conventional x-ray film was fitted and taped to each dried chromatogram. Each chromatogram with film was placed in a light-proof exposure holder which was weighted with a carton of sand to equalize the pressure distribution. The x-ray film was exposed to the chromatogram for 96 hours, then developed

TABLE 1 CHROMATOGRAPHIC RF VALUES OF KNOWN CONTROLS USING A
 50-25-25 MIXTURE OF N-BUTANOL, ACETIC ACID, WATER
 AFTER 14 HOURS DEVELOPMENT

SAMPLE	RF
Fructose-1, 6-Diphosphate	45
Phosphoenolpyruvate	56
2-Phosphoglycerate	53
Hypophosphite	77
Phosphite	57
Phosphate	43

using an automatic rapid processing unit. After exposure the chromatogram was then oxidized and detection completed.

Table 2 shows the results of the autoradiograms and chromatograms for the labeled procedures. As can be seen, the uninoculated culture medium contained a single spot of hypophosphite which showed a high degree of labeled phosphorus. The filtrate showed other phosphorus spots with approximately the same R_f value as phosphate, indicating that the hypophosphite had been partially converted to phosphate. The cell lysate showed five spots of varying degrees of intensity. One spot (a) shows little intensity and has the same R_f value as phosphate. This low intensity of phosphate is consistent with our other investigations which result in accumulations of very low levels of phosphate. Another labeled spot (c) of high intensity shows an R_f value similar to phosphite and 2-phosphoglycerate. This spot has not been identified but it demonstrates that hypophosphite has been converted to another form in the cell. The hypophosphite spot (e) shows an appreciable decrease in intensity indicating that it is indeed being utilized (the uninoculated control showed no decrease in intensity). Another spot (b) has the same R_f value as fructose-1, 6-diphosphate. The final spot (d) has an R_f value different from our control chemicals.

This study does not specifically identify the compounds into which the labeled hypophosphite was converted, but it does demonstrate that hypophosphite is incorporated into intermediate compounds in cellular metabolism, possibly to intermediates of the Emden-Meyerhoff pathway. It also demonstrates release of small amounts of labeled phosphate.

TABLE 2 RF VALUES FROM (³²P) LABELED CHROMATOGRAM AND AUTORADIOGRAPH

SAMPLE	#1	RF	#2	INTENSITY 1-5
HYPOPHOSPHITE	73		75	5
PHOSPHITE	52		53	5
PHOSPHATE	43		43	5
FILTRATE	(A)74;(B)43;(C)40	(A)73;(B)43;(C)40	(A)4;(B)1;(C)1	
MEDIUM	73	73	73	5
CELL LYSATE	(A)42	43	1	1
	(B)45	45		2
	(C)52	52		5
	(D)66	66	2	
	(E)75	75	3	

Because most previous experiments had been performed using hypophosphite as the phosphorus source, another experiment was performed to verify that phosphite could also be utilized by our isolate during anaerobic growth. The basal culture medium used was described in Report No. 7 and sodium phosphite was added to give 100 ppm P. The phosphate level of the culture medium was 0.086 ppm P as phosphate. Replicate bottles of this medium were inoculated with 0.2 ml of a water-washed suspension of the hypophosphite utilizing Bacillus described in Report No. 7. These were incubated at 24°C in Brewer Anaerobe Jars with GasPaks. Duplicate samples were removed at various time intervals, formalin was added to kill the organisms, and the samples were filtered through a 0.22 μ m filter. A portion of the filtrate was analyzed for total phosphorous, then the remainder was separated by column chromatography and analyzed to determine the concentration of phosphite and phosphate. Procedures for chromatographic separation and phosphorous analyses were given in Report No. 7. The pH of the solution, the dry cell weights, glucose concentration, and turbidity were also determined. Uninoculated controls were run with each experimental group.

The results of this investigation are shown in Table 3. As can be seen, there was scant growth, which is characteristic of this isolate on this basal medium. However, the decrease in percent transmittance, the decrease in pH, the decrease in glucose concentration, and the increase in cell weight are all indicative of growth. The depletion of phosphite in the growing samples demonstrates that phosphite is being utilized as the phosphorous source during

TABLE 3 MEASUREMENT OF VARIOUS GROWTH PARAMETERS DURING
ANAEROBIC UTILIZATION OF PHOSPHITE BY A *BACILLUS* SP.

<u>TIME (HRS.)</u>	<u>PPM PHOSPHITE*</u>	<u>%I</u>	<u>pH</u>	<u>CELL WEIGHT (G)</u>	<u>GLUCOSE (%)</u>
T ₀	101	100	7.0	0	.76
T ₂₄	100	100	6.9	1.5x10 ⁻³	.72
T ₅₂	98	98	6.5	4.3x10 ⁻³	.64
T ₇₅	96	95	6.2	7.9x10 ⁻³	.52
T ₉₉	94	89	3.5	9.3x10 ⁻³	.51
CONTROL (140)	101	100	6.7	3.0x10 ⁻⁴	.71

*ONLY PHOSPHOROUS PEAK DETECTED BY COLUMN CHROMATOGRAPHY

anaerobic cultivation. Separation of phosphorous compounds by column chromatography resulted in no free phosphate, and this is consistent with our earlier data. It is postulated that the phosphite is converted into phosphate and incorporated into cellular metabolism, being removed when the cells are filtered. Phosphate released upon the death of a cell would be rapidly incorporated into living cells, thus accounting for the absence of free phosphate in the filtrate.

A similar experiment was designed to determine whether the Bacillus isolate preferred phosphite or hypophosphite as a phosphorous source. This was set up in similar fashion to the preceding experiment with the exception that 60 ppm P of both phosphite and hypophosphite were added to the basal medium. Replicate bottles of media were then inoculated with 0.5 ml of a water-washed suspension of the Bacillus isolate. Aliquots were immediately removed for T_0 analysis of phosphorous and various growth parameters, and the inoculated bottles were then placed into anaerobe jars for incubation at 24°C. Replicate samples were removed periodically, killed, filtered, and analyzed. Phosphorous analysis was again by column chromatography and phosphorous assays of the separated samples. Measurement of growth parameters included those of the preceding experiment plus cell counts. Counts were performed using standard dilution techniques, pour-plates with Trypticase Soy Agar, and anaerobic incubation of the plated samples at 32°C.

The results of this investigation are given in Table 4. These results demonstrate growth of the Bacillus isolate at a rate consistent with previous experiments. The isolate grows only relatively well in

TABLE 4 ANAEROBIC UTILIZATION OF PHOSPHOROUS AND MEASUREMENT
OF GROWTH PARAMETERS OF A CAPE CANAVERAL SOIL ISOLATE

SAMPLE (HOURS)	μg PHOSPHORUS/ML AS $\text{H}_2\text{PO}_2^- - \text{HPO}_3^{2-}$	PH	% GLUCOSE	% TURBIDITY A	PLATE COUNT	CELL WEIGHT (GRAMS)
T ₀	67.5	66.75	.81	100	0	$6.8 \times 10^{-5}^*$
T ₂₄	67	66	.80	100	0	4.6×10^6
T ₄₈	67	63	.78	98	0.009	5.0×10^7
T ₇₂	65	58	.74	95	0.022	5.7×10^7
T ₉₆	63	57	.61	73	0.032	9.3×10^7
T ₁₂₀	-	-	5.8	.73	92	1.8×10^8
T ₁₆₈	63	53	4.8	.66	90	1.5×10^7
T ₁₉₂	-	-	4.9	.64	91	7.5×10^5

* AVERAGE OF 4 PLATES.

the basal medium. It is noted, however, that it grows quite well on TSA on the plate counts, both aerobically and anaerobically. The restricted growth then is a result of the basal medium preparation and not an inherent characteristic of the isolate. The data demonstrates a consistent rate of growth with a corresponding decrease in available phosphorous. It can be seen that the phosphite levels decrease to a greater extent than do the hypophosphite levels, thus indicating that the organism prefers the more oxidized form of phosphorous. These results are seen more graphically in Figure 1 where the actual cell numbers are plotted along with phosphorous concentrations. Although it is not shown in the table or the figure, the phosphate level was also determined in this experiment, with its concentration being virtually unchanged throughout the duration of incubation. The initial phosphate concentration was 0.02 ppm P as phosphate, and this level was maintained throughout the experiment. Again, lack of accumulation of phosphate in the medium is in agreement with all of our phosphorous investigations with this isolate. The growth parameters and phosphorous levels in the uninoculated controls were virtually unchanged for the duration of this experiment.

Another experiment was performed in which phosphate, phosphite, and hypophosphite were all added as phosphorous sources. The results showed slightly better growth with depletion of phosphate, but the phosphite and hypophosphite levels were unchanged. In other words, this isolate has the capability to convert phosphite and hypophosphite to phosphate, but does not do so in the presence of phosphate.

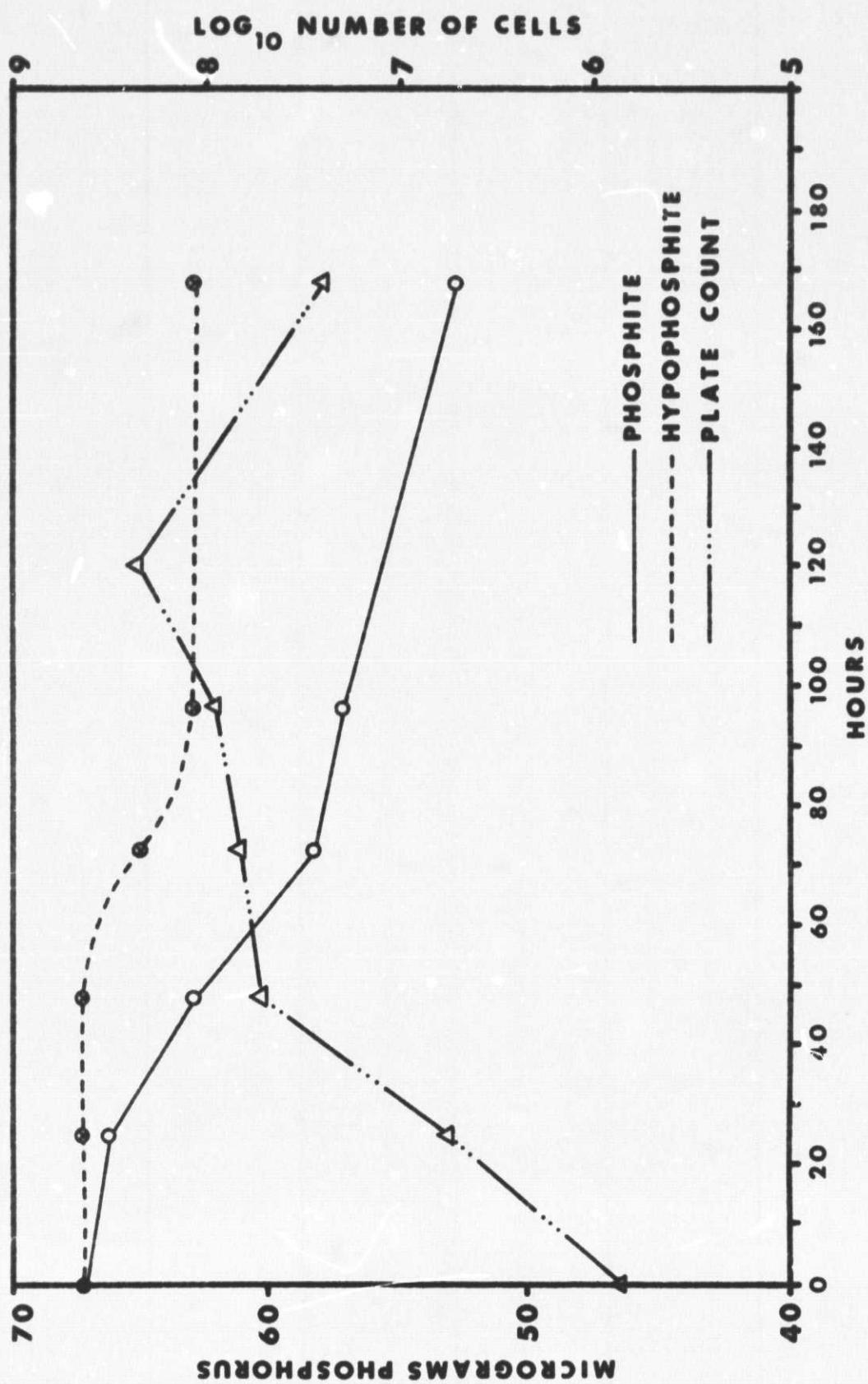


FIGURE 1 CHANGES IN PHOSPHITE AND HYPOPHOSPHITE CONCENTRATIONS DURING ANAEROBIC GROWTH OF A *BACILLUS* SP. ISOLATE FROM CAPE CANAVERAL

CHARACTERIZATION OF ANAEROBIC PHOSPHITE-UTILIZING BACILLUS

This organism was originally isolated through use of the enrichment culture technique in an attempt to isolate from Cape Canaveral soil samples, organisms capable of utilizing phosphite anaerobically. It was isolated on the first attempt and has been reisolated on subsequent attempts. Each isolation procedure has resulted in a pure culture, and cultural, staining, and biochemical characterizations demonstrate that it is the same isolate. Because of its repeated isolation and because of the limited nutrients in the original basal medium, it is assumed that this is a rather common organism in Cape Canaveral soil, and that it is not nutritionally fastidious.

On Trypticase Soy Agar (TSA) colonies appear small, off-white, circular, entire, smooth, opaque, and slightly raised. Subsurface colonies are lenticular, and broth cultures show uniform turbidity throughout the medium. The isolate is a gram-positive rod of varying size in a 24 h culture, and a gram-positive rod of uniform size in a 72 h culture. It produces circular to ellipitical spores which are primarily subterminal, with a few central. The sporangium is not swollen. It produces spores readily on TSA, many spores being present after 24 h incubation at 32°C. Spore suspensions of high titers can be easily prepared by culturing on A-K No. 2 Sporulation Agar.

This isolate was originally isolated under anaerobic conditions, but it also grows very well aerobically. Triplicate tests were all positive for catalase production, so it is a Bacillus sp. Various

biochemical tests were inoculated in quadruplicate, incubated at 32°C and read over a period of 2-21 days, depending on the particular test. Test procedures of Ruth Gordon and of the JPL-PQ lab at Cape Canaveral were employed. The results are presented in Table 5. As can be seen, the isolate is quite active biochemically. Because the results do not readily fit available identification schemes, speciation of this isolate will not be attempted until further tests are performed.

TABLE 5: Results of Quadruplicate Biochemical Tests on Bacillus sp.
Capable of Utilizing Phosphite Anaerobically.

<u>Biochemical Test</u>	<u>Test Number</u>				<u>Incubation Period (days)</u>			
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
PR Mannitol	+	+	+	+	2	2	2	2
Xylose	+	+	+	+	2	2	2	2
Dextrose	+	+	+	+	2	2	2	2
Anaerobic Growth	+	+	+	+	3	3	6	7
Motility	-	-	-	-	6	6	10	14
Saboraud's Dextrose Agar	+	+	+	+	2	2	2	2
Tyrosine	+	+	+	+	4	4	5	5
Gordon's Citrate	+	+	+	+	5	5	5	5
Nitrate Reduction	+	+	+	+	3	3	7	7
Starch Hydrolysis	+	+	+	+	3	3	7	7
V-P	-	-	-	-	4	5	7	7
pH of V-P Medium	4.5	4.5	4.5	4.5	4	5	7	7
Casein Hydrolysis	-	-	-	-	7	7	14	14
Litmus Milk	A ¹	AR ²	AR	AR	4	7	14	14
Phenylalanine	-	-	-	-	5	7	14	21
Growth in 5% NaCl	-	-	-	-	7	7	7	7
Growth in 7% NaCl	-	-	-	-	7	7	7	7

1. A=Acid

2. R=Litmus reduction

OMNITHERMS

In our previous two reports a group of organisms capable of growth over a broad temperature range has been described. These were originally isolated while performing a bacterial soil population profile of Cape Canaveral soil, and all that have been isolated thus far have proven to be members of the genus Bacillus. Since the isolation of these organisms, we have attempted to better characterize their physiological and biochemical properties in an effort to describe their significance to planetary quarantine. In the initial work, this characteristic appeared to be quite stable, and the organisms were grown at 3°*C*, 32°*C*, and 55°*C* on several occasions by different investigators. However, it soon appeared that the storage temperature of the stock cultures has an effect on the stability of this characteristic, some losing their ability to grow at 55°*C*, others losing the ability to grow at 3°*C*. In order to better coordinate results, most of the following work was performed by two groups of investigators, and both groups experienced similar loss of the organisms' capability to grow at all three temperature ranges. For this reason, a great deal of the time of this reported period of investigation has been spent attempting to culture and store these organisms in such a way as to enable them to maintain or again demonstrate this characteristic. These attempts are still in progress, and the optimum storage condition to maintain the omni-therm behavior as a stable characteristic has not yet been determined.

A. Resistance of Spore Suspensions of Omnitherms to the Viking Dry Heat Cycle

The original 28 cultures of omnitherms were grown on TSA plates, washed with sterile phosphate buffer (pH 7.0), heat-shocked at 80°C for 15 minutes, then swabbed onto A-K #2 Sporulating Agar (BBL). These were incubated at 32°C for 5 days and examined for endospores. Five of these did not grow, but the others showed fair to very good spore formation. These were again washed with deionized water, heat-shocked, and replated on A-K Agar for incubation for 5 days at 32°C. After microscopic examination these were washed 6x with sterile phosphate buffer, then 3x with sterile 95% ethanol, and stored in sterile 95% ethanol. These were then titered with results showing from 10^3 to 10^8 spores per ml.

One tenth milliliter of each spore suspension was inoculated into four sterile, screwcap vials, and these were left open under laminar flow until the alcohol had evaporated. They were capped and shipped to John Puleo of the JPL-PQ Lab at Cape Canaveral. Three vials of each spore suspension were subjected to the Viking dry-heat cycle (111.7°C for 30 hours), the fourth vial of each served as a shipping control to determine if there were population changes due to shipping. After heating, the vials were again capped and returned to H-SU for assay by standard plate-count procedures, and the results are presented in Table 6. Of the 23 samples subjected to the dry-heat cycle, 7 showed surviving populations in the 10^2 - 10^3 range, 6 showed surviving populations in the 10^1 range, 5 showed 1 or 2 sterile vials with only small populations in the remaining vial or vials, and only 5 resulted in all sterile vials. Obviously, the spore load in the

TABLE 6: Spore Titers of Omnitherms Before and After Exposure
to Viking Dry-Heat Sterilization Cycle (111.7°C for 30 hrs.)

Isolate	Original Titer ¹ (spores/ml)	Titer of Unheated Control Vial	Titer After Heating
A-28	5.2×10^7	1.1×10^8	1.9×10^1
AA-6	1.5×10^9	2.6×10^8	4.8×10^2
AA-12	1.5×10^5	1.5×10^4	1.1×10^2
BB-1	1.3×10^7	2.7×10^7	0
G-20	2.4×10^7	2.5×10^7	5.3×10^0
G-28-2	6.5×10^7	1.3×10^8	8.4×10^0
G-33	5.2×10^7	1.3×10^8	3.7×10^1
G-33II-1	4.6×10^6	Not Done	2.1×10^1
G-33II-2	4.5×10^7	1.1×10^8	3.3×10^0
G-38A	4.1×10^6	4.7×10^6	9.3×10^0
G38B	8.0×10^8	5.2×10^8	1.9×10^1
G-38C-2	3.1×10^7	8.1×10^7	5.3×10^0
GG-30	2.7×10^8	6.1×10^7	1.4×10^2
GG-41	3.8×10^7	3.1×10^7	5.1×10^3
GG-44	2.3×10^8	1.1×10^8	4.7×10^1
H-86-1	3.7×10^8	1.2×10^8	1.7×10^2
HH-1	6.9×10^6	3.3×10^7	2.9×10^3
HH-1-B	1.6×10^4	3.1×10^4	-
HH-34	3.0×10^7	2.3×10^8	3.9×10^2
HH-66	7.8×10^6	1.2×10^7	0
J-21	1.5×10^8	1.4×10^8	2.2×10^1
JJ-29	2.0×10^3	2.0×10^1	0
JJ-30B	3.3×10^6	1.3×10^6	0

¹All counts are an average of 3 vials (or 6 plates)

vials is much more dense than what will be encountered on a space-craft which has been assembled under clean-room conditions, but these data indicate that some of the omnitherms possess a fair degree of dry-heat resistance. For example, isolates AA-12 and HH-1 show only a 3 log population decrease after 30 hours at 111.7°C.

Seven of the omnitherms which survived the dry-heat cycle were inoculated in duplicate onto TSA slants and TSB to determine if they were still capable of growing at 3°, 32°, and 55°C. This should give some indication as to whether or not this trait is destroyed by extreme temperatures. The growth in TSB was very poor, which is a characteristic which has been noted on other occasions. Organisms growing at the extreme temperatures always seem to grow better on agar than in broth. For this reason, the results presented in Table 7 are for growth on TSA slants. This characteristic has prompted us recently to initiate investigations to determine the most suitable culture medium for culturing the omnitherms.

As can be seen from Table 7, five of the seven heat-treated isolates grew at all three temperatures during the incubation periods used. The 3°C slants showed growth in 7-9 days, with those not showing growth being observed for 14 days. All of the 32°C slants showed growth in 2 days, and the 55°C slants showed growth in 2-3 days. It appears that this omnithermic characteristic is not destroyed by heating (111.7°C for 30 h). The only change observed is that the heat-treated isolates grew better and faster at 55°C than had been observed previously.

TABLE 7 RELATIVE GROWTH OF SEVEN OMNITHERMS AT
DIFFERENT TEMPERATURE RANGES

ISOLATE	20°C	32°C	35°C	38°C
AA-12	+1*	+1	+3	
G-33A	0	+2	+3	
GG-30	+1	+2	+1	
GG-41	+1	+3	+2	
H-86-1	+1	+2	+3	
HH-34	0	+2	+3	
J-21	+1	+2	+3	

*A SCALE WAS ARBITRARILY ESTABLISHED FROM SCANT (+1) TO ABUNDANT (+4) GROWTH.

B. Some Physiological Characteristics of Omnitherms

Certain physiological properties of microorganisms are of special interest to planetary quarantine. One such property is the ability of the organisms to grow under anaerobic conditions. All of the omnitherms which we have been able to maintain on stock cultures grow aerobically, even though some were originally isolated under anaerobic conditions. An experiment was performed to determine how many of these could also grow anaerobically at the three different temperatures used in our work.

Because these routinely demonstrate better growth on TSA, slants were prepared and stored for 24 hours in a Brewer anaerobe jar with GasPak to deoxygenate the medium. Three slants per culture per temperature were inoculated for anaerobic incubation at 3⁰C, 32⁰C, and 55⁰C. A single aerobic control per culture per temperature was also inoculated, and uninoculated slants were also included as controls at each temperature. Cultures at 3⁰C were examined for growth after 7 days, at 10 days, and at 3 day intervals thereafter. Cultures at 32⁰C were examined in 48 hrs., and those at 55⁰C were examined after 3 days and at 3 day intervals thereafter. A colony from an 18-24 hr. culture of each isolate incubated on TSA plates at 32⁰C was suspended in 2.0 ml of 0.1% peptone, and one drop of this suspension was used as the inoculum for each slant. The results of facultative growth of these organisms are shown in Table 8.

Another characteristic of significance to planetary quarantine is the salt tolerance of microorganisms. In order to evaluate this for the omnitherms, we prepared TSB tubes with salt (NaCl) concentrations from 1 through 10%, 12%, and 15%. TSB was used in this

TABLE 8. Anaerobic Growth of Omnitherms at 3°, 32°, and 55°C
and Maximum Salt Concentration at Which Growth Occurs (Aerobically at 32°C)

<u>Isolate</u>	<u>Anaerobic Growth</u>			<u>Maximum Salt Concentration(%)</u>
	<u>3°C</u>	<u>32°C</u>	<u>55°C</u>	
A-28	0 ¹	+1	0	12
AA-6	0	+2	0	12
AA-10	0	+1	0	12
AA-12	0	+2	0	12
BB-1	0	+1	+2	8
C-16	0	+1	+1	12
G-20	+2	+1	+1	6
G-28-2	0	+1	0	12
G-33	+1	+1	0	12
G-33II-1	0	+2	+1	12
G-33II-2	+1	+1	0	ND
G-38A	0	+1	+1	12
G-38B	0	+1	+1	12
G-38C	0	+1	+1	12
GG-30	0	+3	+1	7
GG-41	0	+1	0	12
GG-44	0	+1	0	4
H-86-1	NG	NG	NG	10
HH-1	+1	+1	0	15
HH-1A	0	0	+1	12
HH-1B	+1	+2	0	12
HH-34	0	+2	0	12
HH-66	0	+1	0	10
J-21	0	+1	0	12
JJ-28-1	0	+3	0	9
JJ-28-2	0	+2	0	9
JJ-29	0	+3	0	10
JJ-30A	0	+2	0	15
JJ-30B	NG	NG	NG	5

1 Arbitrary comparison of growth from none (0) to heavy (+4)

2 ND=Not Done

3 NG=Non-viable culture (no growth, even in controls)

experiment because all incubations were performed aerobically at 32° C. Duplicate tubes of each salt concentration were inoculated with one drop of a 0.1% peptone suspension of each isolate. These were incubated for 48 hrs., then examined for turbidity. Uninoculated tubes of the different salt concentrations were used as negative controls in this experiment. The results of this investigation are also shown in Table 8.

As can be seen from Table 8, anaerobic growth of most of these isolates is quite sparse. The tests were performed in triplicate and results where growth could not be clearly ascertained were reported as negative. It is known that temperature does affect the ability of an organism to grow in the presence of or absence of oxygen, and these results verify this. All isolates are capable of anaerobic growth at at least one of the temperatures employed, with approximately half of them growing anaerobically at only 32° C. About one-fourth of them grow anaerobically at 32° and 55° C, and about 14% of them grow anaerobically at 3° and 32° C. Only one isolate (G-20) showed anaerobic growth at all three temperatures.

Again from Table 8, it can be seen that all of these isolates grew in salt concentrations of up to 4%. This should not be surprising because these were originally isolated from soil samples from Cape Canaveral. In addition, 21 of the 28 isolates were capable of growth up to at least 10% NaCl, and 18 of 28 grew in salt concentrations of 12%. Only two of the isolates grew in a 15% salt concentration.

These results, in addition to the omnithermic characteristic, again demonstrate the tremendous adaptability of this particular group

of organisms. They may be of special significance to planetary quarantine research because of their ability to grow over broad temperature ranges, to grow aerobically and anaerobically at different temperatures, to grow in high salt concentrations, and because they appear to possess an appreciable degree of dry-heat resistance.

C. Growth Curves of Omnithermic Isolate

Five isolates were streaked onto TSA plates and incubated at 32°*C* for 24 hrs. to isolate individual colonies. Colonies were transferred to 10 ml TSB and again incubated at 32°*C* for 24 hrs. One-tenth ml aliquots were used to inoculate 3 flasks containing 250 ml TSB per organism. Samples were immediately taken from each flask for N_0 counts, and the flasks were incubated aerobically at 3°, 37°, and 55°*C*. At various time intervals, the flasks were sonicated for 2 min, and 5.0 ml samples were removed. One ml of this was used for counting, the remaining four ml being used for turbidometric measurements. All counts were performed by diluting the sample in 0.1% peptone and employing the pour-plate procedure with TSA as the culture medium. These triplicate plates were incubated at 37° for 48 hrs. prior to counting.

Growth curves for isolate G-20 at the three temperatures are shown in Figure 2. As can be seen, populations at all temperatures shows an initial decline, especially at 55°*C*, followed by a fairly rapid increase. This isolate appears to grow very well at all three temperatures, reaching maximum population in 19 days at 3°*C*, in 23 hrs. at 55°*C*, and still increasing after 24 hrs. at 37°*C*.

Data is also available for drawing standard curves of

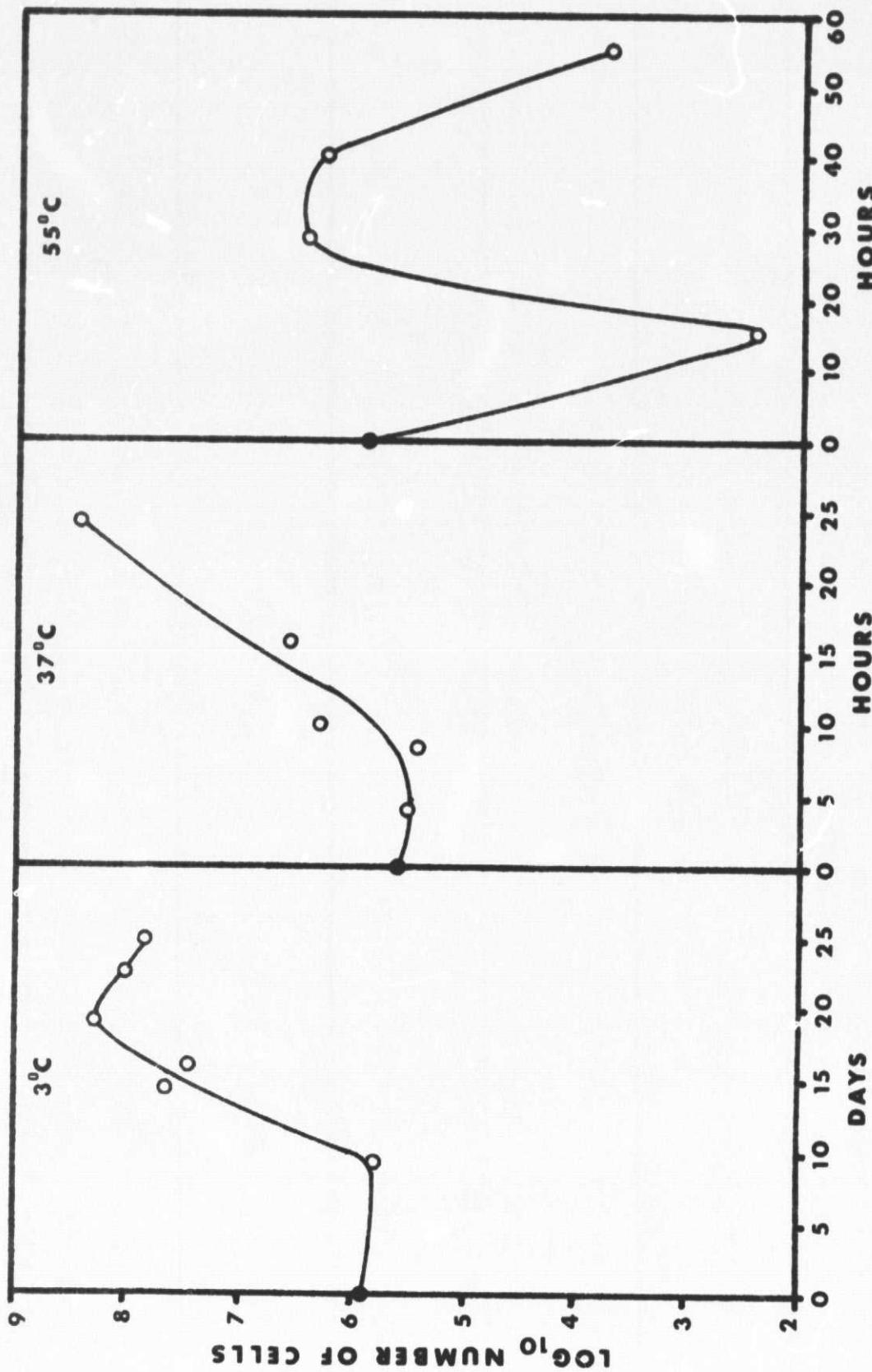


FIGURE 2 GROWTH CURVES FOR ISOLATE G-20 AT THREE DIFFERENT TEMPERATURES

turbidity vs. viable cell count, but those curves are not presented in this report. From the growth curves presented, generation times for G-20 at the three different temperatures have been calculated and are 21 hrs. for 3°^oC, 1.8 hrs. for 37°^oC, and .9 hrs. for 55°^oC.

In a separate investigation another of our research groups has begun determination of generation times at 3°, 32°, and 55°^oC for 28 of these isolates. In this procedure sidearm flasks are inoculated as described above, then incubated at the three different temperatures. These are periodically sonicated in an ultrasonic bath for one minute, and the turbidity in the sidearm is read on a Klett-Summerson photometer. Growth curves for the isolates can then be drawn by plotting Klett units vs. time, and generation times can be calculated from this data. By using this procedure, it is possible to handle larger numbers of samples at multiple temperatures incubation.

This procedure has recently been performed on 28 isolates at 32°^oC and viable cell counts are being performed on some of these to verify the efficacy of this procedure. The data acquired thus far will not be presented in this report, but when the efficacy has been verified, this procedure will be performed on the various isolates at 3°, 32°, and 55°^oC, and possibly other temperatures. These data should be available for the next report.

D. Second Attempt to Isolate Omnitherms from Cape Canaveral Soil Samples

The original isolation of omnitherms in our lab was performed on a mixed soil sample from Cape Canaveral. This mixture consisted of samples from the vicinity of the Vehicle Assembly Building (VAB),

Spacecraft Assembly and Encapsulation Facilities (SAEF 1 and 2), launch complex 41, and the fuel storage area. The results of this work are given in Report 6. Since that time the mixed soil sample used in that experiment has been stored in a sealed plastic bag in a 4°C refrigerator.

In a separate, long-term experiment, samples of the same soil sample mixture were stored in plastic cups with air-tight seals at -65°C and at 24°C to evaluate the effect of long term storage on total soil populations. The results of this experiment are also in Report 6 and show that the total population remained relatively constant for the 160-day duration of the experiment. In addition, the thermophilic level remained unchanged, while the psychrophilic level show a slight decrease with storage of the sample at 24°C.

In this present investigation, these same three soil samples were used to enumerate bacterial populations at 3°, 32°, and 55°C. It is noted that the samples have been in storage (-65°C, 4°C, and 24°C) for 12-15 mo. hs at the time this present investigation was performed.

Duplicate soil samples were serially diluted in 0.1% peptone and plated to yield triplicate plates for each sample at each temperature. These were incubated for 14 days at 3°C, 48 hrs. at 32°C, and 48 hrs. at 55°C prior to counting. The results are given in Table 9. By comparing these counts back to the data presented in Report 6, it can be seen that the aerobic, mesophilic counts of these soil samples are virtually identical to those of one year ago. This does not speak to the question of type of organisms present, but the total populations are unchanged after prolonged storage. It can also be determined

TABLE 9 BACTERIAL COUNTS OF A MIXED CAPE CANAVERAL SOIL SAMPLE
AFTER PROLONGED STORAGE AT DIFFERENT TEMPERATURES

SOIL STORAGE TEMPERATURE	INCUBATION TEMPERATURE	PLATE COUNT	AVERAGE COUNT
			(CFU* / G. SOIL)
-65°C	30°C	6.6x10 ⁴ **	6.6x10 ⁴ **
	32°C		1.0x10 ⁷
	35°C		5.4x10 ⁵
24°C	30°C	6.1x10 ⁴	6.1x10 ⁴
	32°C		1.4x10 ⁷
	35°C		4.2x10 ⁵
4°C	30°C	9.1x10 ³	9.1x10 ³
	32°C		5.4x10 ⁶
	35°C		2.1x10 ⁵

* COLONY FORMING UNITS
** AVERAGE OF 6 PLATES

that the thermophilic counts are virtually the same in each sample as they were in Report 6, whereas the psychrophilic counts show a decrease of about 1 log in all cases.

Countable plates from each soil sample and each incubation temperature were selected for replicate plating. The replicate plating procedure employing straight pins, as described in Report 7, was used to replicate colonies isolated at one temperature to the other two temperatures. Another replicate plate was reincubated at the original isolation temperature to serve as a control. By recording growth of each colony at the three different temperatures, it was possible to calculate a bacterial population profile based upon temperatures of growth. These results are presented in Table 10. Again, it can be seen that omniphiles were isolated from all three soil samples, even though the percentage appears lower than that found in the original work (omniphiles 3.4% - see Table 2, Report 6). This apparent difference could be the result of several factors. The soil samples are much older, and the total number of colonies examined is larger. However, a very likely difference is in replication techniques. In the original work, colonies were picked individually with an inoculating needle and transferred to TSA slants. This was a slow, laborious process, and the plates necessarily aged more. The pin-replicate plating technique is much faster, and the plates do not get nearly as old. This is reflected in the viability of the cultures. In the previous work 11.4% of the colonies did not grow on subculture. In the present investigation, loss of viability ranged from 0.4% to 1.5%. Had the original work shown such a high degree of viability, the percentage of omnitherms could have been decreased accordingly.

TABLE 10 POPULATION DISTRIBUTION OF CAPE CANAVERAL SOIL SAMPLES AFTER PROLONGED STORAGE AT DIFFERENT TEMPERATURES (GIVEN IN PERCENTS)

<u>ORGANISM TYPE</u>	<u>-65°C</u>	<u>SOIL STORAGE TEMPERATURE 24°C</u>	<u>40°C</u>
PSYCHROPHILE	0	0	0
FACULTATIVE PSYCHROPHILE	50.4	25.7	52.1
MESOPHILE	42.5	66.6	38.0
FACULTATIVE THERMOPHILE	6.2	6.4	6.4
THERMOPHILE	0	0.1	0.1
OMNITHERM	0.5	0.3	0.7
NON-VIABLE	0.4	1.0	1.5
TOTAL COLONIES REPLICATED	744.0	1140.0	949.0

Table 10 also shows that no obligate psychrophiles were isolated whereas 1.4% were found in the original investigation. Prolonged storage of the soil samples does appear to favor a change in the population distribution of the sample. Another noteworthy change is the fact that the sample stored at 24°C showed a decrease in the facultative psychrophile population with a resultant increase in the mesophile population. The two samples stored at low temperatures exhibited relatively little change in these categories.

PLANS FOR FUTURE ACTIVITIES

Investigations concerning anaerobic utilization of phosphite and hyphophosphite have been completed. Equipment has been received and set up, and protocol have been developed to investigate anaerobic utilization of phosphine. In this investigation, the same basal medium is being used and phosphine gas is bubbled through it to supply the phosphorous source. Phosphine gas spontaneously ignites in the presence of oxygen, thus requiring not only anaerobic incubation of the samples, but complete handling of samples and media under anaerobic conditions. Systems have been designed for this process, and we can currently work with multiple samples simultaneously. Enrichment culture techniques have already been initiated to isolate organisms which can utilize phosphine anaerobically, and we are currently processing samples in the third subculture. Soil samples from various areas of Cape Canaveral are being used, and at least four of them continue to show turbidity after about two weeks incubation in the phosphine atmosphere. If isolates are indeed found which appear capable of utilizing phosphine, then experiments similar to those with phosphite will be performed to verify this phenomenon. Analytical methods for monitoring phosphine concentration have already been developed and verified with known controls.

Because it has been suggested that phosphine may be toxic to microorganisms, experiments have been initiated to determine phosphine toxicity to various types of microorganisms and to spore suspensions. Preliminary results show no decrease in bacterial populations in the presence of phosphine.

Emphasis on studies concerning omnitherms will be decreased after the next report period. Because of the dry-heat resistance of these isolates, they will be investigated in conjunction with the JPL-PQ lab at Cape Canaveral. They will be examined with the pyrolysis-gas chromatography equipment in an attempt to determine if there is any relationship between these and the "hardy" organisms described by Puleo. Another effort will be to determine the most suitable culture medium for cultivation of omnitherms, and attempts will be made to determine the optimum storage conditions for omnitherms to assure that they maintain the ability to grow over broad temperature ranges.

A new endeavor will be initiated in the very near future by our lab. Because of the anaerobic conditions of the Jovian planets, an investigation of anaerobic microbiology associated with spacecraft environments appears warranted. This phase of work will be concerned with the distribution of anaerobic microbes in such areas, but, more importantly, attempts will be made to develop new or improved techniques for performing such work.